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13. ABSTRACT (Maximum 200 Words)

We are studying anastellin, a novel anti-angiogenic protein. We have made significant progress toward understanding the mechanism of action of anastellin, and of polymeric fibronectin, the formation of which is induced by anastellin. We have shown that anastellin is ineffective in mice lacking plasma fibronectin, whereas the activity of the anti-angiogenic form of antithrombin does not depend on plasma fibronectin. As antithrombin is known to bind to another plasma adhesion protein, vitronectin, we also tested anastellin and antithrombin in vitronectin null mice. Antithrombin is inactive in these mice, whereas anastellin is active. Strikingly, a third anti-angiogenic protein, endostatin, was poorly active both in the plasma fibronectin-deficient and vitronectin null mice (Yi et al., *PNAS*, in press). These results provide strong evidence for our original hypothesis, which predicted that the various anti-angiogenic compounds depend on adhesion proteins, such as fibronectin and vitronectin, for their activity. Drawing from this new understanding of the mechanism of action, we are currently designing anastellin variants with enhanced activities and studying the effects of anastellin on endothelial cells *in vitro*. These advances in mechanistic understanding will help in designing experimental anti-angiogenic treatments for ovarian cancer.

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INTRODUCTION

We have developed a novel substance, a polymeric form of fibronectin that we have named sFN, which has profound anti-tumor activities. Importantly, there seems to be no toxicity associated with systemic sFN treatment, even when given over several months. At least two characteristics of sFN contribute to its anti-tumor effects: it is both anti-angiogenic and anti-metastatic. sFN is a complex of a fragment from fibronectin (which binds tightly to fibronectin) and of fibronectin itself. The sFN-inducing fragment, which we have named 'anastellin', reproduces the anti-tumor effect of sFN (Yi and Ruoslahti, 2001). Anastellin is thought to form a functional sFN complex with fibronectin present in blood. There are striking similarities between anastellin and the other known anti-angiogenic compounds, which are heparin binding and have the ability to make complexes with adhesion proteins that interact with the $\alpha v\beta 3$ integrin. The function of this integrin is essential to the survival of angiogenic endothelial cells.

In the original application, we proposed to experimentally test the hypothesis that sFN and many of the known protein inhibitors of angiogenesis share a mechanism of action: They form complexes with adhesion proteins in plasma and the resulting complexes then somehow selectively suppress angiogenic endothelial cells, most likely through the $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ integrins, which are specifically expressed in angiogenesis.

We have provided strong proof for this hypothesis as detailed below. Because sFN is particularly effective in suppressing the growth and spreading of experimental ovarian cancer in mice, and clinical trials are planned in this cancer, these studies are relevant to cancer in general and ovarian cancer in particular.

BODY

The approved tasks for this project were:

Task 1: To develop conditions for preparing (1) antithrombin-vitronectin complexes, (2) fibrinogen complexes made in a manner analogous to the preparation of sFN, and (3) sFN at different degrees of polymerization.

Task 2: To study the effects of the above reagents on endothelial cell proliferation, apoptosis and migration, and angiogenesis on chorioallantoic membrane (CAM).

Task 3: To test the most promising materials from Task 2 for inhibition of tumor growth in a mouse ovarian cancer model.

Task 1. We have made particularly significant progress with Task 1; our findings may be a major advance in the anti-angiogenic treatment field. We had previously shown that anastellin causes polymerization of fibronectin and fibrinogen. Under this grant, we have now shown that anastellin requires fibronectin to be active *in vivo*. We used "conditional knockout" mice that lose their ability to produce fibronectin in the liver and, as a consequence, have little or no plasma fibronectin (Sakai *et al.*, 2001). The assay was based on inhibition of angiogenesis in subcutaneously implanted plugs of basement membrane material (matrigel), which had been impregnated with angiogenic factors.

We found that anastellin was completely inactive in these plasma fibronectin-deficient mice. As these mice do have fibrinogen, these experiments answer one of the questions we posed; the fibrinogen-polymerizing activity of anastellin does not seem to be relevant to the anti-angiogenic activity of anastellin. Hence, we have not pursued further fibrinogen work. We extended the analysis to include mice that lack another adhesion protein abundant in plasma, vitronectin (Zheng *et al.*, 1995). Anastellin was fully active in these mice. The anti-angiogenic form of antithrombin showed the opposite pattern; it does not depend on plasma fibronectin, but is inactive in the vitronectin null mice. Strikingly, a third anti-angiogenic protein, endostatin, was poorly active both in the plasma fibronectin-deficient and vitronectin null mice. These new results (Yi et al., 2003) provide a strong validation for our plan to study matrix protein complexes.

The results described above also provide the kind of guidance we originally proposed to obtain for the development of the most efficient anti-angiogenic compound for the treatment of experimental ovarian cancer. We had originally proposed to improve the efficiency of fibronectin polymerization by anastellin. Because endostatin is, on a molar basis, more active than anastellin as an anti-angiogenic compound, comparison of the two compounds gives useful information on how to optimize anti-angiogenic activity. While endostatin requires plasma fibronectin for activity, it does not detectably polymerize fibronectin. We initially thought it did, but further studies failed to confirm that. We were unable to test the binding of endostatin to fibronectin or vitronectin because endostatin gives a high background in binding assays. We hypothesize that some unknown molecule(s) may mediate an interaction of endostatin with fibronectin and vitronectin.

The lack of detectable fibronectin polymerization by endostatin suggested that we should reduce, not enhance, the polymerizing activity of anastellin to develop more efficient anastellin compounds. We have prepared a set of anastellin variants that differ in their abilities to polymerize fibronectin (Briknarová et al., 2003). Matrigel assays show that these variant anastellins also differ in their anti-angiogenic activities *in vivo*. Variants that do not interact with fibronectin are inactive, those with low polymerizing activity are more active than wild-type anastellin, and those that polymerize better than anastellin are less active (Akerman et al., unpublished results).

Task 2. We are conducting *in vitro* studies on the effects of anastellin and anastellin-fibronectin complexes on endothelial cells. These studies are incomplete, but several new observations have emerged (Pilch et al., unpublished). These results indicate that there is a receptor for anastellin and that anastellin has a cytotoxic/proapoptotic activity. We will continue exploring that anastellin mechanism of action under other funding.

A network of fibrillar fibronectin surrounds cells and provides survival signals to them. Treatment of endothelial cells with anastellin in vitro results in degradation of the fibronectin matrix, suppressed cell proliferation, and activation of the p38 mitogen-activated protein kinase, a signaling molecule that may also be involved in apoptosis induced by cell stress (Bourdoulous et al., 1998).

We have analyzed cell viability of cells treated with anastellin in conjunction with different types of cell stress. We found that Chinese hamster ovary cells exposed to amino acid starvation prior to anastellin treatment undergo cell death at a significantly higher level than cells cultured in normal medium. This effect was even more pronounced when anastellin was mixed with fibronectin to yield sFN (Fig. 1). Incubation of HUVEC with cobalt chloride, a compound known to mimic hypoxia, also showed synergy with anastellin (Fig. 2). Interestingly, pretreatment with cobalt chloride also enhanced anastellin binding to HUVEC, which otherwise bound little anastellin as measured by flow cytometry (Fig. 3).

These results suggest that anastellin is cytotoxic under defined conditions and provides us with an *in vitro* system in which to study the mechanisms of anastellin activity. Future studies will focus on: (i) the supporting effect of cell stress, (ii) the regulation of the anastellin binding site on cells, (iii) anastellin-induced cell signals, and (iv) the cofactor function of plasma fibronectin.

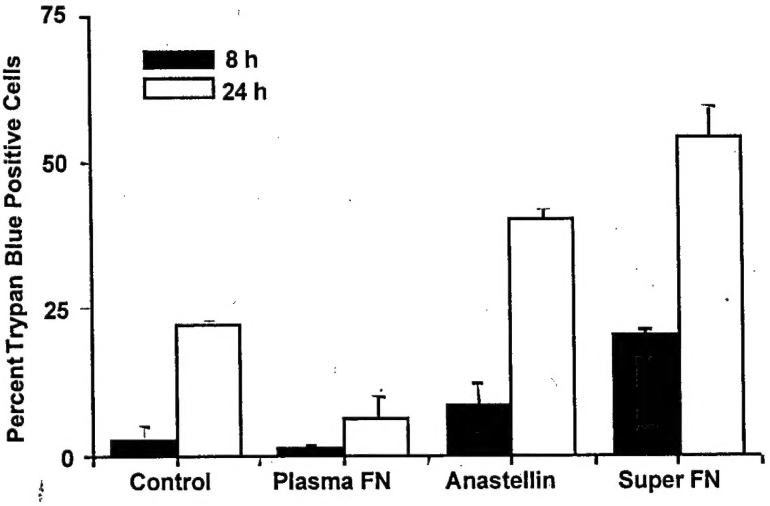

Fig. 1. Anastellin induces cell death. CHO cells stressed by amino acid starvation were treated with anastellin or a mixture of anastellin and fibronectin (Super FN) for the indicated times. Cell death was measured by trypan-blue exclusion assay. Fibronectin alone (Plasma FN) had a slight protective effect.

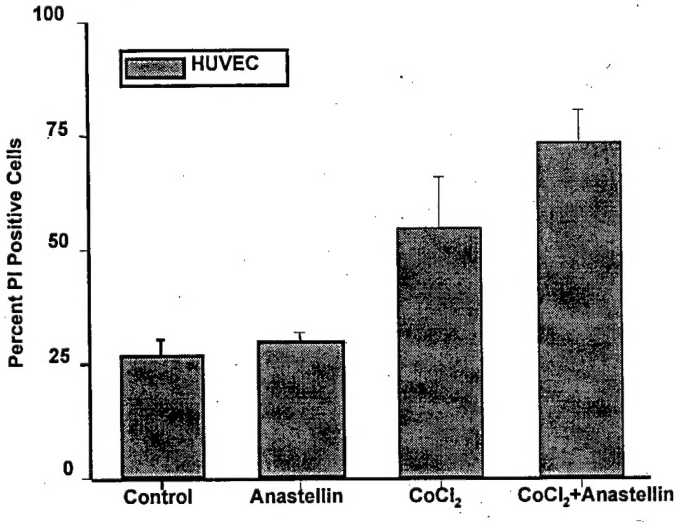

Fig. 2. Hypoxia induced by cobalt chloride treatment enhances the cytotoxity of anastellin to HUVEC. Cell death was assessed by counting propidium iodide-positive (PI) cells in flow cytometry.

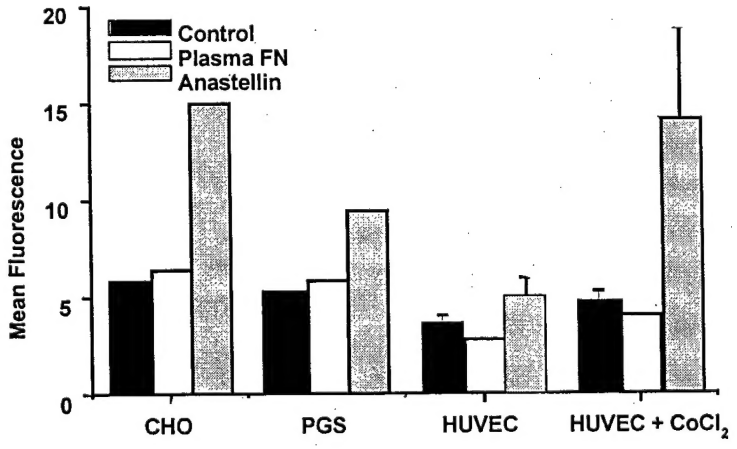

Fig. 3. Anastellin binds to cells. Cobalt chloride treatment enhances the binding. Various cell lines were treated with $CoCl_2$ or left untreated prior to incubation with anastellin, fibronectin, or buffer as a control. Cell binding was measured by detecting bound anastellin or fibronectin with anti-fibronectin antibodies in flow cytometry.

Task 3. The striking new results we have obtained under Task 1 have changed our thinking with regard to what might be an optimal design for an anastellin-based anti-angiogenic compound, and those studies are still in progress. Consequently, we have postponed entering into the tumor treatment experiments. However, the advances we have made in mechanistic understanding will help in designing anti-angiogenic treatments for ovarian cancer. For example, any intraperitoneal treatment with anti-angiogenic proteins will have to take into consideration the availability of fibronectin and vitronectin in the peritoneal cavity. In our ovarian cancer treatment experiments, we combined anastellin with fibronectin. Addition of the appropriate adhesion protein to the treatment protocol may be necessary with other anti-angiogenic proteins as well. The new information and new anastellin variant we have produced will allow optimization of the treatment in experimental animals.

KEY RESEARCH ACCOMPLISHMENTS

We have demonstrated the following:

- Anastellin is ineffective in mice that lack plasma fibronectin but active in mice that lack vitronectin.
- Antithrombin inhibits angiogenesis in mice that lack plasma fibronectin, but is inactive in mice that lack vitronectin.
- Endostatin activity is greatly impaired both in the plasma fibronectin-deficient and vitronectin null mice.
- Mutation of certain critical residues in anastellin can enhance its anti-angiogenic activity.

Our results strongly support our hypothesis regarding the mode of action of the various anti-angiogenic compounds. They all appear to form complexes with adhesion proteins that contain RGD sequences. These complexes may bind to the integrins on the endothelial cells of angiogenic tumor vessels, stopping proliferation and inducing apoptosis. Now that adhesion protein complexes have been shown to be crucial to the activities of 3 (out of 3) anti-angiogenic proteins, other anti-angiogenic compounds should be studied for a similar dependence on adhesion proteins. Our results also point to the importance of understanding the exact role of the integrins that are specifically expressed on angiogenic endothelial cells.

REPORTABLE OUTCOMES

Yi, M., Sakai, T., Fässler, R., and Ruoslahti, E. Anastellin, endostatin, and antithrombin require plasma fibronectin or vitronectin for anti-angiogenic activity. *Proc. Natl. Acad. Sci. USA.*, in press (2003).

CONCLUSIONS

We have made significant progress toward understanding the mechanism of action of anastellin (the III1-C fibronectin fragment). We have confirmed the previous preliminary result that anastellin is ineffective in mice lacking plasma fibronectin. The new important results are that endostatin

appears to be inactive in these plasma fibronectin-negative mice and that endostatin, like anastellin, can polymerize fibronectin. This activity of endostatin is new. Moreover, antithrombin is inactive in mice that lack vitronectin. As the anti-angiogenic form of antithrombin is known to bind to vitronectin, this result is exactly what our original hypothesis predicted. This result suggests that fibronectin polymerization is important for anastellin and endostatin activity. Antithrombin apparently forms similar complexes with plasma vitronectin.

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Yi, M., Sakai, T., Fässler, R., and Ruoslahti, E. Anastellin, endostatin, and antithrombin require plasma fibronectin or vitronectin for anti-angiogenic activity. *Proc. Natl. Acad. Sci. USA.*, in press (2003)

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APPENDIX COVER SHEET

Antiangiogenic proteins require plasma fibronectin or vitronectin for *in vivo* activity

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Contributed by Erkki Ruoslahti, August 8, 2003

Fragmentation of various extracellular matrix and blood proteins generates antiangiogenic substances that are physiological regulators of angiogenesis. Some of these compounds are in clinical trials as inhibitors of tumor angiogenesis. Anastellin, an antiangiogenic protein fragment derived from fibronectin, was unable to inhibit matrigel plug angiogenesis in mice that lack plasma fibronectin. Anastellin was fully active in mice that are null for vitronectin, which, like fibronectin, is a major adhesion protein in the blood. An antiangiogenic form of antithrombin showed the opposite pattern. The activity of endostatin was impaired in both fibronectin- and vitronectin-deficient mice. These results suggest a shared mechanism of action for antiangiogenic factors derived from extracellular matrix and plasma proteins: these factors form complexes with adhesion proteins in plasma to create an active antiangiogenic substance.

A growing class of antiangiogenic substances is derived from extracellular matrix and blood proteins by proteolysis or other modifications. These substances include fragments from thrombospondin (1), plasminogen (angiostatin; ref. 2), collagen type XVIII (endostatin; ref. 3), collagen type IV (tumstatin; ref. 4), a modified form of antithrombin (5), and the fibronectin fragment anastellin (6, 7). The molecular mechanisms whereby these substances exert their antiangiogenic activities are poorly understood. Anastellin binds to and polymerizes fibronectin and fibrinogen (8, 9). The antiangiogenic form of antithrombin is similar to the modified antithrombin that binds vitronectin (10, 11). Vitronectin, like fibronectin, is an arginine-glycine-aspartic acid (RGD)-containing adhesion protein present in plasma (12). Other angiogenesis inhibitors also interact with one or more adhesion proteins: angiostatin and its parent protein, plasminogen, bind vitronectin (13), whereas endostatin binds fibulins and nidogen-2 (14).

The interactions of the various angiogenesis inhibitors with adhesion proteins led us to hypothesize that adhesion protein binding could underlie antiangiogenic activity, and that activities of the various inhibitors could converge on this ability to form such adhesion protein complexes (7). To test this hypothesis, we examined the two antiangiogenic proteins with the most extensively documented adhesion protein interactions, anastellin and antithrombin. We used angiogenesis induced by implanting matrigel (basement membrane) plugs (15, 16) into mutant mice that conditionally lack plasma fibronectin (17) or have their vitronectin gene knocked out (18).

We show here that anastellin does not suppress angiogenesis in matrigel plugs implanted into mice that lack plasma fibronectin. In contrast, the antiangiogenic form of antithrombin is inactive in mice that lack vitronectin. Endostatin is essentially inactive in mice that lack either plasma fibronectin or vitronectin. These results strongly support the hypothesis that the activity of extracellular matrix- and blood protein-derived angiogenesis inhibitors depends on interactions with adhesion proteins.

Materials and Methods

Proteins. Anastellin (a fragment from the first type III repeat in human fibronectin) and III11-C (control fragment from the 11th type III repeat of fibronectin) were prepared as recombinant his-tagged proteins in bacteria and purified as described (6, 17). Human plasma fibronectin and human vitronectin were from Chemicon (Temecula, CA), and human fibrinogen was from Sigma. The protein solutions were sterilized by filtering through a 0.2-μm membrane before experimentation. Recombinant human endostatin and human plasma antithrombin III were from Calbiochem. Antithrombin III was treated with guanidine·HCl to convert the native protein to its antiangiogenic form as described (5). This form of the protein is henceforth referred to as antithrombin.

Mice. Two-month-old immunodeficient female BALB/c*nu/nu* mice (Harlan–Sprague–Dawley, San Diego) were used to establish the model for matrigel angiogenesis inhibition. Two plasma fibronectin *Cre-loxP* conditional knockout mouse lines [Fn(fl/fl); *Alb-Cre*+, and Fn(fl/fl); *Mx-Cre*+] (17) were used. In the Fn(fl/fl); *Alb-Cre*+ mice, the Cre expression is under the control of the albumin promoter and causes postnatal elimination of the fibronectin gene in the liver, which is the source of essentially all plasma fibronectin. The Fn(fl/fl); *Mx-Cre*+ line expresses Cre in an IFN-inducible manner. Deletion of the fibronectin gene in the mice was induced in 4- to 5-week-old mice as described (17). These mice have been shown to express <0.04% of the normal plasma fibronectin level (17). A vitronectin-null mouse line (18) was kindly provided by David Loskutoff (The Scripps Research Institute, La Jolla, CA). Wild-type C57BL/6J mice were used as controls for the vitronectin knockout mice. All knockout mice were genotyped. Immunoblotting performed at the time the mice were used in the experiments (4 months of age) showed no detectable fibronectin in the plasma of the mice with deleted fibronectin genes; their plasma vitronectin levels were normal. The vitronectin knockout mice were negative for plasma vitronectin, but had normal plasma fibronectin levels (results not shown).

Matrigel Angiogenesis Assay. Matrigel was from Becton Dickinson. Recombinant human basic fibroblast growth factor (bFGF) and recombinant mouse vascular endothelial growth factor (VEGF) were from R & D Systems. The rat anti-mouse CD31 antibody was from Pharmingen. Liquid matrigel containing 100 ng of bFGF or 50 ng of VEGF per ml was injected s.c. in the abdominal region of the mouse. Each mouse received one, or usually two, 0.5-ml matrigel plugs.

The mice were treated with daily i.p. injections of one of the angiogenesis inhibitors in 0.3 ml of PBS, or PBS as a control. A fragment corresponding to the homologous residues from the 11th type III domain of human fibronectin was used as a treatment control for anastellin (6). After 7–10 days, the mice were killed and the matrigel plugs were removed. The matrigel plugs were fixed in 4% paraformaldehyde, stored in 70% etha-

Abbreviation: bFGF, basic fibroblast growth factor.

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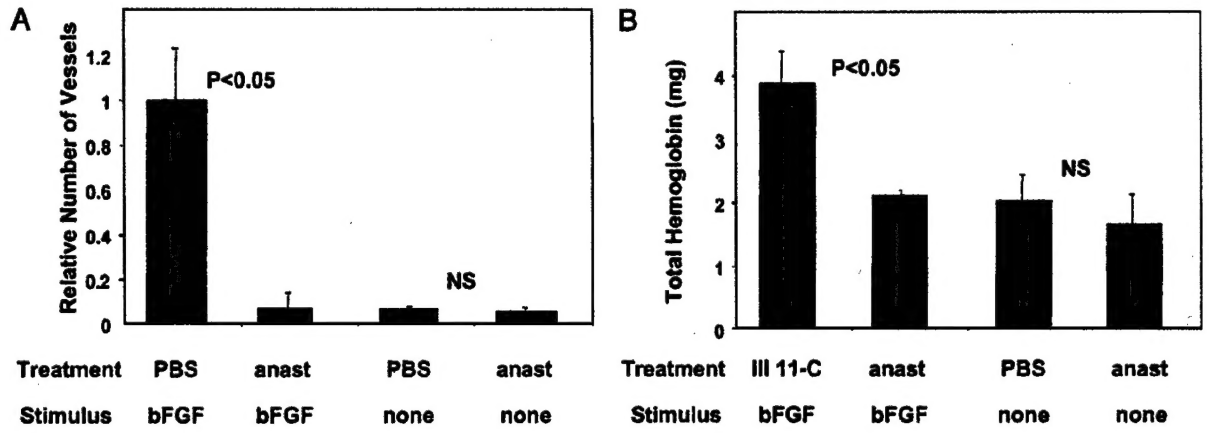

Fig. 1. *In vivo* angiogenesis assay in matrigel plugs. Mice bearing matrigel plugs impregnated with bFGF were treated with daily i.p. injections of 1 mg of anastellin (anast), PBS, or a control fragment homologous to anastellin from the 11th type III domain of fibronectin for 10 days. Angiogenesis was evaluated by counting the number of blood vessels in the plugs (*A*) or by measuring the hemoglobin content in duplicate plugs (*B*). Combined results from three independent experiments, with three or four mice in each test group, are shown. The *P* values show the significance level of the differences observed between the indicated test groups. NS, not significant.

nol, and used to cut sections. Paraffin embedding, sectioning, and immunostaining of the plugs for CD31 and other blood vessel markers were carried out in The Burnham Institute's Histology Facility or at Pharmingen. Blood vessels were counted in an average of three sections from each matrigel plug. Alternatively, the plugs were homogenized and their hemoglobin content was determined by using the Drabkin reagent kit (Sigma). Student's *t* test was used in statistical analysis of the results.

Results

To validate the matrigel model, we implanted mice with s.c. matrigel plugs that were supplemented with either bFGF or vascular endothelial growth factor, and treated the mice with i.p. injections of anastellin. The number of blood vessels and total hemoglobin content in the plugs correlated with the amount of the angiogenic factor added to the gel. Anastellin treatment essentially eliminated the angiogenic effect of both substances, but had no effect on the basal level of blood vessel formation in plugs that received no growth factor. A control fragment analogous to anastellin, but derived from another fibronectin type III (11th type III) domain, was inactive. Based on the results exemplified in Fig. 1, we chose 50 ng of bFGF as the angiogenic stimulus for the testing of the adhesion protein dependence of three angiogenesis inhibitors: anastellin, antithrombin, and endostatin.

Anastellin had no antiangiogenic activity in the fibronectin-deficient mice, but was fully active in their normal littermates. The results obtained by determining blood vessel density in tissue sections (Fig. 2) were confirmed by measuring the amount of hemoglobin in the plugs (Table 1). In contrast, anastellin was fully active in vitronectin-null mice (Fig. 2). Thus, plasma fibronectin is necessary for the antiangiogenic activity of anastellin, but vitronectin is not.

Antithrombin modified by denaturation or proteolysis becomes an angiogenesis inhibitor (5) and gains the ability to bind vitronectin (10, 11). Denatured antithrombin inhibited angiogenesis in wild-type mice and in the plasma fibronectin-deficient mice, but was inactive in the vitronectin-null mice. Blood vessel counts and hemoglobin values were highly significant (Fig. 3 and

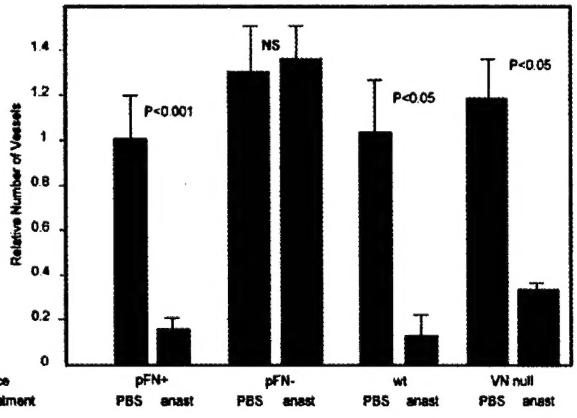

Fig. 2. Anastellin activity requires plasma fibronectin, but not vitronectin. Mice deficient in plasma fibronectin (pFN−), their littermate controls (pFN+), vitronectin-null mice (VN null), and same strain wild-type (wt) controls for the VN null mice were injected with matrigel plugs impregnated with bFGF. The mice were then treated with seven daily i.p. injections of 1 mg of anastellin or PBS. The number of blood vessels in the plugs is shown. The two fibronectin-deficient lines gave similar results; combined results from three experiments with 56 mice are shown. Twelve mice were used to study the role of vitronectin. The *P* values show the significance level of the differences observed between the indicated test groups. NS, not significant.

Table 1. Effect of angiogenesis inhibitors on the hemoglobin content of matrigel pellets in mice lacking plasma fibronectin or vitronectin

	Mouse line			
Treatment	pFN+	pFN−	wt	VN null
PBS	8.6 ± 1.2	7.2 ± 0.8	5.1 ± 0.2	6.3 ± 0.8
Anastellin	2.5 ± 0.3**	7.6 ± 1.2	2.0 ± 0.2**	2.4 ± 0.6**
PBS	7.8 ± 0.7	7.2 ± 0.4	6.2 ± 0.4	6.5 ± 0.4
Antithrombin	3.8 ± 0.6**	4.0 ± 0.7**	2.3 ± 0.1**	6.0 ± 0.3
PBS	7.9 ± 0.7	7.3 ± 0.8	7.3 ± 1.1	5.9 ± 0.9
Endostatin	3.6 ± 0.5**	6.8 ± 0.8	2.6 ± 0.5**	3.9 ± 0.6

The hemoglobin content (in mg, average ± SEM) in duplicate matrigel plugs removed from the treatment experiments in Figs. 2, 3, and 5 is shown. **$P < 0.01$.

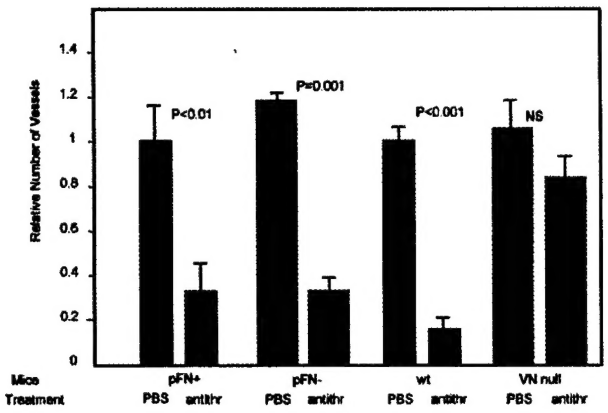

Fig. 3. Antithrombin is active in plasma fibronectin-deficient mice but is inactive in vitronectin-null mice. Mice with matrigel plugs were treated with seven daily i.p. injections of 270 μg or 180 μg of antithrombin (antithr) or PBS, as in Fig. 2. The number of blood vessels in the plugs is shown. Results from mice treated with the two doses were pooled (16 mice in fibronectin experiments; 24 mice in vitronectin experiments).

Table 1). Fig. 4 shows representative micrographs of matrigel plugs from the antithrombin-treated and control mice; these results show that vitronectin is required for the antiangiogenic activity of antithrombin.

We also tested the dependence of the antiangiogenic activity of endostatin on plasma fibronectin and vitronectin. Like anastellin, endostatin is an angiogenesis inhibitor that is derived from an extracellular matrix protein. Endostatin was strongly antiangiogenic in plasma fibronectin-positive control mice and in wild-type mice, but its activity was impaired in fibronectin-deficient and vitronectin-null mice. The data suggested partial remaining activity in the mutant mice, but the difference to the PBS control was not statistically significant (Fig. 5). Thus, endostatin requires both fibronectin and vitronectin for full activity.

Discussion

Our results suggest a common mechanism of action for protein inhibitors of angiogenesis: they form protein complexes with RGD-containing plasma adhesion proteins such as fibronectin or vitronectin. The binding specificity of anastellin for fibronectin (6, 9) and of modified antithrombin (5) for vitronectin (10, 11) was reflected in the dependence of their angiogenic activity on plasma fibronectin and vitronectin, respectively. That endostatin required both plasma fibronectin and vitronectin to be antiangiogenic *in vivo* was unexpected. Endostatin is not known to directly bind to fibronectin or vitronectin, but does bind to fibulin and nidogen (14). Fibulin, which is present in plasma at a concentration of ≈30 μg/ml (19), interacts with fibronectin (20). Endostatin (and anastellin and antithrombin) binds to heparin, and fibronectin has several heparin-binding sites (3, 5, 9, 21). Thus, one or more other proteins or proteoglycans could mediate a binding of endostatin to fibronectin and vitronectin in plasma or at cell surfaces. Angiostatin can bind vitronectin (13), as does at least one other antiangiogenic factor, the matricellular protein, SPARC (22). Thus, endostatin, angiostatin, and SPARC also have the potential of forming complexes with adhesion proteins in plasma.

The $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ integrins, which are selectively expressed in angiogenic vessels (23, 24) are likely targets of the antiangiogenic protein/adhesion protein complexes. Both fibronectin and vitronectin are ligands for the $\alpha v\beta 3$ integrin. Fibronectin also binds to $\alpha 5\beta 1$, and vitronectin to $\alpha v\beta 5$ (25). Endostatin binds to $\alpha 5\beta 1$ (26) and tumstatin to $\alpha v\beta 3$ (4), and these integrins mediate the *in vitro* effects of endostatin and tumstatin on endothelial cells (4, 27). Gene knockout experi-

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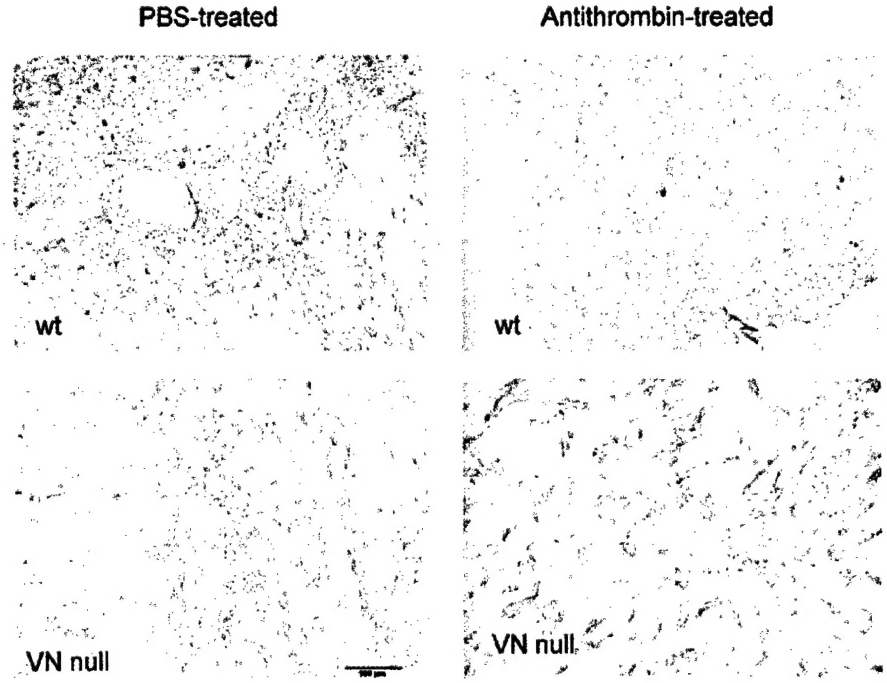

Fig. 4. Reduced blood vessel density in matrigel plugs from mice treated with antithrombin. Representative micrographs showing CD31-stained vessels in matrigel plugs of mice from four test groups in Fig. 3 are shown. (Magnification, ×400.)

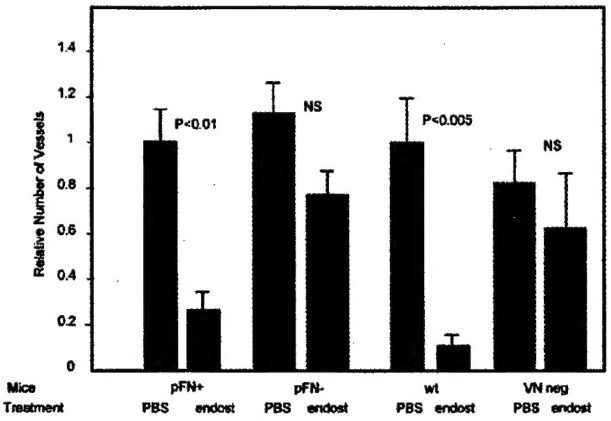

Fig. 5. Endostatin antiangiogenic activity is impaired in plasma fibronectin-deficient and vitronectin-null mice. Mice with matrigel plugs were treated with seven daily i.p. injections of 120 μg of endostatin (endost) or PBS, as in Fig. 2. The number of blood vessels in the plugs from a total of 24 mice is shown.

ments show that $\alpha5\beta1$ is necessary for vascular development (28). The vasculature develops and angiogenesis takes place in mice that lack $\alpha v\beta3$ or all αv integrins (29, 30), but in an adult animal, perturbing the function of either $\alpha5\beta1$ or $\alpha v\beta3$ causes endothelial cell apoptosis and inhibits angiogenesis (23, 24, 31). Moreover, synthetic RGD peptide polymers that mimic polymeric adhesion proteins can inhibit angiogenesis (32). The mechanism of endothelial damage by the antiangiogenic complexes remains to be elucidated. One possibility is that soluble complexes may elicit different integrin signals than the attachment of cells to a substrate, which promotes survival. Accumulation of RGD-containing peptides in the cytoplasm has been reported to initiate apoptosis (33, 34); internalization and proteolysis of antiangiogenic factor/adhesion protein complexes could provide a supply of cytoplasmic RGD peptides. Alternatively, the integrin-binding complexes could bind to circulating endothelial precursor cells (35), preventing them from contributing to the formation of new blood vessels. The hypothesis that RGD-directed integrins on angiogenic endothelial cells would be the common target of adhesion protein complexes assembled by the antiangiogenic proteins, provides a unifying explanation for the activities of a bewildering number of diverse antiangiogenic factors.

In addition to being relevant to the understanding of the control of angiogenesis, our results may allow antiangiogenic compounds to be used more effectively in the clinic. For example, some cancer patients who have received chemotherapy have low fibronectin levels (36). An antiangiogenic protein that depends on plasma fibronectin for its activity could be less effective in patients with low fibronectin levels. Little information is available on plasma vitronectin levels in pathological conditions, but similar considerations may apply to antithrombin and other angiogenesis inhibitors that might depend on vitronectin.

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